



FINAL REPORT

Test Facility Study No. 511884

Determination of 'Ready' Biodegradability: Carbon Dioxide (CO₂) Evolution Test (Modified Sturm Test) of MLA-3202

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13 October 2016

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1. STATEMENT OF GLP COMPLIANCE

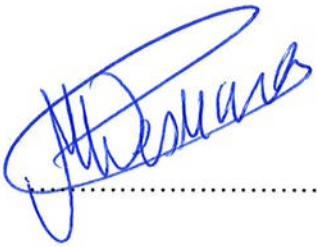
Charles River Den Bosch BV, 's-Hertogenbosch, The Netherlands

All phases of this study performed by the test facility were conducted in compliance with the following GLP regulations:

- OECD Principles of Good Laboratory Practice concerning Mutual Acceptance of Data in the Assessment of Chemicals, 26 November 1997 (C(97) 186 Final);
- EC Council Directive 2004 (2004/10/EC, February 11, 2004, Official Journal of February 20, 2004).

The data generated and reported are considered to be valid.

Charles River Den Bosch

Signature: 

Name: M.J.E. Desmares-Koopmans, Bachelor, ERT

Title: Study Director

Date: 13. October 2016

2. TEST FACILITY QUALITY ASSURANCE STATEMENT

Charles River Den Bosch BV, 's-Hertogenbosch, The Netherlands.

Study title: Determination of 'ready' biodegradability: carbon dioxide (CO₂) evolution test (modified Sturm test) of MLA-3202

This report was inspected by the Charles River Den Bosch Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s).

The reported method and procedures were found to describe those used and the report reflects the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Project 511884

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	Study Plan	31-May-2016	31-May-2016	31-May-2016
	Study Plan Amendment 01	05-Jul-2016	05-Jul-2016	05-Jul-2016
	Study Plan Amendment 02	20-Jul-2016	20-Jul-2016	20-Jul-2016
	Report	26-Sep-2016	26-Sep-2016	26-Sep-2016
Process	Test Substance Receipt	09-May-2016	20-May-2016	24-May-2016
	Test Substance Handling			
	Environmental Science	04-Jul-2016	08-Jul-2016	12-Jul-2016
	Test Substance Handling			
	Exposure			
	Observations/Measurements			

The review of the final report was completed on the date of signing this QA statement.

The facility inspection program is conducted in accordance with Standard Operating Procedure.

Charles River Den Bosch

Signature:

ALI BOUHUIZEN, MSc.

Name: Manager Regulatory Compliance

Date: 07-Oct-2016

3. SUMMARY

Determination of 'ready' biodegradability: carbon dioxide (CO_2) evolution test (modified Sturm test) of MLA-3202.

The study procedures described in this report were in compliance with the OECD guideline No. 301 B, 1992. In addition, the procedures were designed to meet the test methods of the Council Regulation (EC) No. 440/2008 of 30 May 2008, Publication No. L142, Part C.4-C and ISO 9439, 1999 and ISO 10634, 1995.

MLA-3202 was a clear amber-red liquid UVCB. The test item was tested in duplicate at a concentration of 17 mg/L, corresponding to 12 mg TOC/L. For calculation of the organic carbon content the ratio of the components was taken into account. The Theoretical CO_2 production (Th CO_2) of MLA-3202 was calculated to be 2.65 mg CO_2 /mg.

The study consisted of six bottles:

- 2 inoculum blanks (no test item),
- 2 test bottles (MLA-3202),
- 1 positive control (sodium acetate) and
- 1 toxicity control (MLA-3202 plus sodium acetate).

Since MLA-3202 was not sufficiently soluble to allow preparation of an aqueous solution at a concentration of 1 g/L, weighed amounts were added to the 2-litres test bottles containing medium with microbial organisms and mineral components. To this end, 10 mL of Milli-RO water was added to each weighing bottle containing the test item. After vigorous mixing (vortex) the resulting suspension was added quantitatively to the test medium. The test solutions were continuously stirred during the test to ensure optimal contact between the test item and test medium.

The relative biodegradation values calculated from the measurements performed during the test period revealed 50% and 46% biodegradation of MLA-3202 (based on Th CO_2), for the duplicate bottles tested. Thus, the criterion for ready biodegradability (at least 60% biodegradation within a 10-day window) was not met.

In the toxicity control, more than 25% biodegradation occurred within 14 days (50%, based on Th CO_2). Therefore, the test item was assumed not to inhibit microbial activity.

Since all criteria for acceptability of the test were met, this study was considered to be valid.

MLA-3202 was designated as not readily biodegradable.

4. INTRODUCTION

Due to the acquisition of WIL Research by Charles River, the name of the WIL Research facility in Den Bosch, has been changed to Charles River Laboratories Den Bosch BV, Hambakenwetering 7, 5231 DD Den Bosch, The Netherlands. Study documents may contain both names and both names are considered equivalent and may be used as the name of WIL Research transitions to Charles River.

4.1. Study schedule

Experimental starting date : 04 July 2016
Experimental completion date : 04 August 2016

4.2. Purpose

The purpose of the study was to evaluate a non-volatile test item for its ready biodegradability in an aerobic aqueous medium with microbial activity introduced by inoculation with the supernatant of activated sludge.

4.3. Guidelines

The study procedures described in this report are in compliance with the Organization for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, Section 3, Degradation and Accumulation, guideline No. 301 B: "Ready Biodegradability: CO₂ Evolution Test" adopted July 17, 1992.

In addition, the procedures were designed to meet the test methods prescribed by the following guidelines:

Council Regulation (EC) No. 440/2008 of 30 May 2008, Part C: Methods for the determination of ecotoxicity, Publication No. L142, C.4. "Biodegradation: determination of the 'ready' biodegradability, C.4-C: Carbon dioxide (CO₂) evolution test (Modified Sturm Test).

ISO International Standard 9439 "Water Quality - Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium - carbon dioxide evolution test (1999).

ISO International Standard 10634 "Water Quality - Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium" (1995).

4.4. Retention of records and materials

Records and material pertaining to the study, which include study plan and amendments, raw data, specimens, except perishable specimens, and the final report will be retained in the archives of the test facility for a minimum of 5 years after the finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. The test facility will retain information concerning decisions made.

Perishable specimens (e.g. requiring refrigeration or freezing) will be discarded following evaluation in the study without further notice to the study sponsor.

A sample of the test item will be retained until expiry date or applicable retest date. After this period the sample(s) will be destroyed.

4.5. Responsible personnel

4.5.1. Test facility

Study Director M.J.E. Desmares-Koopmans, Bachelor, ERT

4.5.2. Sponsor Representative

Study Monitor Audrey Batoon, PhD

4.6. Definitions

Readily biodegradable are those test items giving a result of at least 60% biodegradation within 28 days. This pass level must be reached within the 10 days immediately following the attainment of 10% biodegradation (10-day window).

Since a test on a mixture of structurally similar chemicals is performed and it is anticipated that a sequential biodegradation of the individual structures is taking place, the 10-day window will not be applied to interpret the results of the test.

Theoretical carbon dioxide (ThCO₂) is the quantity of carbon dioxide calculated (mg) to be produced from the known or measured carbon content of the test item when fully mineralized; also expressed as mg carbon dioxide evolved per mg test item.

Total Organic Carbon (TOC) of a sample is the sum of the organic carbon in solution and in suspension.

5. MATERIALS AND METHODS

5.1. Test item

5.1.1. Test item information

Test item	207258/A
Identification	MLA-3202
Appearance	Clear amber-red liquid
Batch	RC-1045
Purity/Composition	UVCB
Test item storage	At room temperature
Stable under storage conditions until	17 February 2019 (expiry date)

For Certificate of Analysis see [APPENDIX 2](#).

5.1.2. Study specific test item information

Purity/composition correction factor	No correction factor required
Test item handling	No specific handling conditions required
Chemical name (IUPAC), synonym or trade name	Amides, tallow, N,N-bis(2-hydroxypropyl)
CAS Number	1454803-04-3
Specific gravity/density	0.9394
Solubility in water	< 1 g/L
Stability in water	Yes

5.2. Vehicle information

Not applicable.

5.3. Reference item

5.3.1. Reference item information

Identification number	RS186
Container	D1
Identification	Sodium acetate
CAS Number	127-09-3
Molecular formula	CH ₃ COONa
Molecular weight	82.03
Appearance	White powder (determined at Charles River Den Bosch)
Batch	AM0793468
Purity	99.1%
Storage conditions	At room temperature
Stable under storage conditions until	28 February 2017
Supplier	Merck KGaA, Darmstadt, Germany
Article number	1.06268
Certified	Yes

5.3.2. Reference item concentration and preparation of test solutions

A solution of sodium acetate was prepared by dissolving 1003.9 mg in Milli-RO water and making this up to a total volume of 250 mL. Volumes of 20 mL from this stock solution were added to 2 litres of the test medium of the positive control bottle and the toxicity control bottle, resulting in a final concentration of 40 mg sodium acetate per litre (12 mg TOC/L).

5.4. Test concentration and preparation of test solutions

MLA-3202 was a clear amber-red liquid UVCB. The test item was tested in duplicate at a concentration of 17 mg/L, corresponding to 12 mg TOC/L. For calculation of the organic carbon content the ratio of the components was taken into account.

In a pre-test MLA-3202 was not sufficiently soluble to allow preparation of an aqueous solution at a concentration of 1 g/L. Therefore, weighed amounts were added to the 2-litres test bottles containing medium with microbial organisms and mineral components (test item bottle A: 33.1 mg; test item bottle B: 33.1 mg and toxicity control bottle: 33.3 mg). To this end, 10 mL of Milli-RO water was added to each weighing bottle containing the test item. After vigorous mixing (vortex) the resulting suspension was added quantitatively to the test medium. The test solutions were continuously stirred during the test, to ensure optimal contact between the test item and the test organisms.

5.5. Test system

Source	The source of test organisms was activated sludge freshly obtained from a municipal sewage treatment plant: 'Waterschap Aa en Maas', Heeswijk-Dinther, The Netherlands, receiving predominantly domestic sewage.
Treatment	The freshly obtained sludge was preconditioned to experimental conditions by continuous aeration until further treatment. The concentration of suspended solids was determined to be 3.4 g/L in the concentrated sludge. Before use, the sludge was allowed to settle (44 minutes) and the supernatant liquid was used as inoculum at the amount of 10 mL/L of mineral medium.

Reason for selection The test has been accepted internationally for determining the 'ready' biodegradability of test items under aerobic conditions.

5.6. Test procedure and conditions

Test duration	28 days (last CO ₂ measurement on day 29). During the test period, the test media were aerated and stirred continuously.
Test vessels	2 litre glass brown coloured bottles.
Milli-RO water	Tap-water purified by reverse osmosis (Milli-RO) and subsequently passed over activated carbon.
Stock solutions of mineral components	A) 8.50 g KH ₂ PO ₄ 21.75 g K ₂ HPO ₄ 67.20 g Na ₂ HPO ₄ .12H ₂ O 0.50 g NH ₄ Cl dissolved in Milli-RO water and made up to 1 litre, pH 7.4 ± 0.2 B) 22.50 g MgSO ₄ .7H ₂ O dissolved in Milli-RO water and made up to 1 litre. C) 36.40 g CaCl ₂ .2H ₂ O dissolved in Milli-RO water and made up to 1 litre. D) 0.25 g FeCl ₃ .6H ₂ O dissolved in Milli-RO water and made up to 1 litre.
Mineral medium	1 litre mineral medium contains: 10 mL of solution (A), 1 mL of solutions (B) to (D) and Milli-RO water.
Barium hydroxide	0.0125 M Ba(OH) ₂ (Boom, Meppel, The Netherlands), stored in a sealed vessel to prevent absorption of CO ₂ from the air.
Synthetic air (CO ₂ < 1 ppm) ¹	A mixture of oxygen (ca. 20%) and nitrogen (ca. 80%) was passed through a bottle, containing 0.5 - 1 litre 0.0125 M Ba(OH) ₂ solution to trap CO ₂ which might be present in small amounts. The synthetic air was sparged through the scrubbing solutions at a rate of approximately 1-2 bubbles per second (ca. 30-100 mL/min).
Illumination	The test media were excluded from light.
5.6.1. Preparation of bottles	
Pre-incubation medium	The day before the start of the test (day -1) mineral components, Milli-RO water (ca. 80% of final volume) and inoculum (1% of final volume) were added to each bottle. This mixture was aerated with synthetic air overnight to purge the system of CO ₂ .
Type and number of bottles	Test suspension: containing test item and inoculum (2 bottles).

¹ Gas cylinder, Air products, Air zero, O₂ 20.9%±1%, H₂O < 3 ppm, CO+CO₂ < 1 ppm, THC (as CH₄) < 0.2 ppm.

Inoculum blank: containing only inoculum (2 bottles)
Positive control: containing reference item and inoculum (1 bottle).
Toxicity control: containing test item, reference item and inoculum (1 bottle).

Preparation

At the start of the test (day 0), test and reference item were added to the bottles containing the microbial organisms and mineral components.
The volumes of suspensions were made up to 2 litres with Milli-RO water, resulting in the mineral medium described before.
Three CO₂-absorbers (bottles filled with 100 mL 0.0125 M Ba(OH)₂) were connected in series to the exit air line of each test bottle.

5.6.2. Determination of CO₂

Experimental CO₂ production

The CO₂ produced in each test bottle reacted with the barium hydroxide in the gas scrubbing bottle and precipitated out as barium carbonate. The amount of CO₂ produced was determined by titrating the remaining Ba(OH)₂ with 0.05 M standardized HCl (1:20 dilution from 1 M HCl (Titrisol® ampoule), Merck, Darmstadt, Germany).

Measurements

Titrations were made every second or third day during the first 10 days, and thereafter at least every fifth day until day 28, for the inoculum blank and test suspension.
Titrations for the positive and toxicity control were made over a period of at least 14 days.

Each time the CO₂-absorber nearest to the test bottle was removed for titration; each of the remaining two absorbers was moved one position in the direction of the test bottle. A new CO₂-absorber was placed at the far end of the series. Phenolphthalein (1% solution in ethanol, Merck) was used as pH-indicator.

On day 28, the pH of all test suspensions was measured and 1 mL of concentrated HCl (37%, Merck) was added to the bottles of the inoculum blank and test suspension. The bottles were aerated overnight to drive off CO₂ present in the test suspension. The final titration was made on day 29.

Theoretical CO₂ production

The theoretical CO₂ production was calculated from the molecular formula.

5.6.3. Measurements and recording

pH

At the start of the test (day 0) and on day 28, before addition of concentrated HCl.

Temperature of medium

Continuously in a vessel with Milli-RO water in the same room.

5.7. Interpretation

5.7.1. Data evaluation

ThCO₂, expressed as mg CO₂/mg test item, was calculated as follows:

$$\text{ThCO}_2 = \frac{\text{No. of carbon atoms in test item} \times \text{Molecular weight CO}_2}{\text{Molecular weight test item}}$$

For the calculation of the total ThCO₂ the ratio of the components was taken into account.

The first step in calculating the amount of CO₂ produced is to correct for background (endogenous) CO₂ production. Thus the amount of CO₂ produced by a test item is determined by the difference (in mL of titrant) between the experimental and blank Ba(OH)₂ traps.

The amount of 0.05 M HCl titrated is converted into mg of CO₂ produced:

$$\text{mg CO}_2 = \frac{0.05 \times \Delta \text{ mL HCl titrated}}{2} \times 44 = 1.1 \times \Delta \text{ mL HCl titrated}$$

Relative biodegradation values were calculated from the cumulative CO₂ production relative to the total expected CO₂ production, based on the total carbon content of the amount of test item present in the test bottles. A figure of more than 10% biodegradation was considered significant.

The relative biodegradation values were plotted versus time together with the relative biodegradation of the positive control. If applicable, the number of days is calculated from the attainment of 10% biodegradation until 60% biodegradation. Should this period be ≤ 10 days (10-day window), then the test item is designated as readily biodegradable.

Toxicity control: if less than 25% biodegradation (based on ThCO₂ of the test and positive control items combined) occurred within 14 days, the test item was assumed to be inhibitory.

The total CO₂ evolution in the inoculum blank was determined by the cumulative difference (in mL of titrant) between the blank Ba(OH)₂ traps and untreated Ba(OH)₂ (background).

5.7.2. Acceptability of the test

1. The positive control item was biodegraded by at least 60% (73%) within 14 days.
2. The difference of duplicate values for %-degradation of the test item was always less than 20 ($\leq 4\%$).
3. The total CO₂ release in the blank at the end of the test did not exceed 40 mg/L (53.7 mg CO₂ per 2 litres of medium, corresponding to 26.9 mg CO₂/L).
4. The Inorganic Carbon content (IC) of the test item (suspension) in the mineral medium at the beginning of the test was less than 5% of the Total Carbon content (TC). Since the test medium was prepared in tap-water purified by reverse osmosis (Milli-RO water (Millipore Corp., Bedford, Mass., USA, carbon levels < 500 ppb)), IC was less than 5% of TC (mainly coming from the test item, 12 mg TOC/L).

Since all criteria for acceptability of the test were met, this study was considered to be valid.

5.8. List of deviations

5.8.1. List of study plan deviations

1. A temporary breakdown in the aeration (< 1 day) was noted on day 15.
Evaluation: This relative short breakdown was considered to have no effect on the outcome of this study.

The study integrity was not adversely affected by the deviations.

5.8.2. List of standard operating procedures deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

6. ELECTRONIC SYSTEMS FOR DATA ACQUISITION

The following electronic systems were used for data acquisition:

- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA): Temperature.

7. RESULTS

7.1. Theoretical CO₂ production

The ThCO₂ of MLA-3202 was calculated to be 2.65 mg CO₂/mg (for details on the calculation see [Table 1](#)).

The ThCO₂ of sodium acetate was calculated to be 1.07 mg CO₂/mg.

If applicable the ThCO₂ per test bottle are given in the subscript of the tables (see [APPENDIX 1](#)).

Table 1
ThCO₂ calculation of MLA-3202.

Percent Composition		Formula		MW	%C:	ThCO ₂ :		
33.1	99.4	C ₂₄	H ₄₇	N ₁	O ₃	397.6	0.72	2.66
22.9		C ₂₂	H ₄₅	N ₁	O ₃	371.6	0.71	2.61
13.6		C ₂₄	H ₄₅	N ₁	O ₃	395.6	0.73	2.67
11		C ₂₄	H ₄₉	N ₁	O ₃	399.6	0.72	2.64
6		C ₂₂	H ₄₃	N ₁	O ₃	369.6	0.71	2.62
3.2		C ₂₆	H ₄₅	N ₁	O ₃	419.6	0.74	2.73
2.0		C ₂₄	H ₄₃	N ₁	O ₃	393.6	0.73	2.68
1.5		C ₁₈	H ₃₄		O ₂	282.4	0.77	2.81
1.1		C ₂₆	H ₄₇	N ₁	O ₃	421.6	0.74	2.71
5.6	5.6							
100.0						0.72	2.65	

7.2. Biodegradation

All data are presented in [APPENDIX 1](#). The results of CO₂ production and biodegradation in blank bottles, background bottles and each test bottle are listed in [Table 3](#) to [8](#). [Table 10](#) contains the comparison of biodegradation of MLA-3202 in bottles A and B.

Figure 1 shows the curves for biodegradation of the two bottles with MLA-3202, the positive control and the toxicity control.

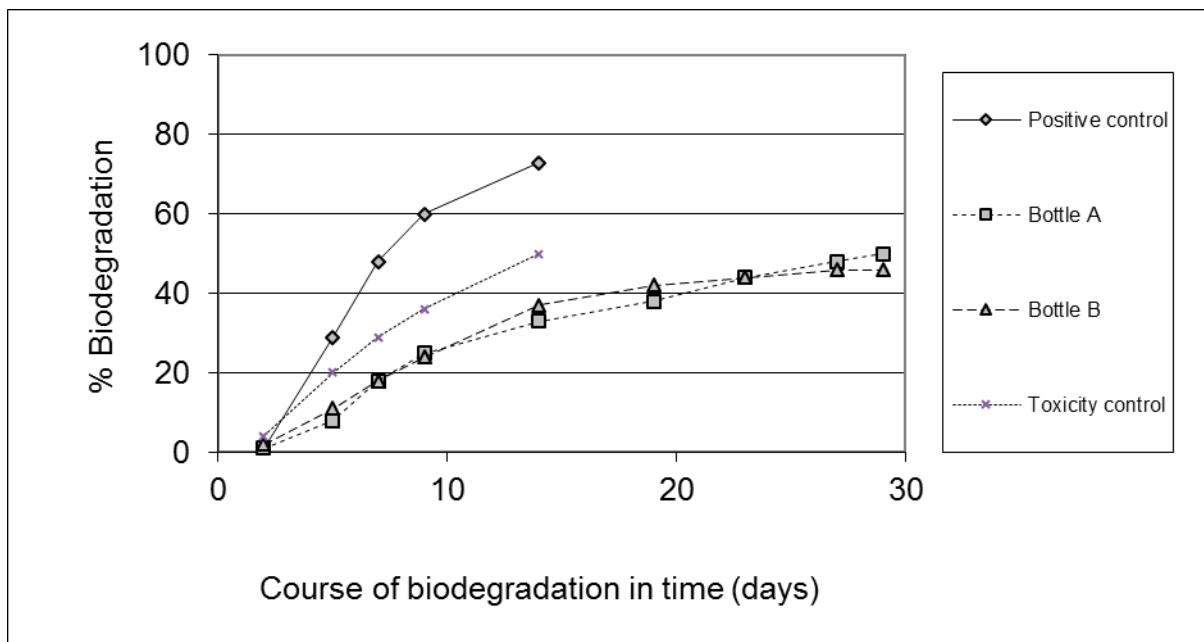


Figure 1 Biodegradation of MLA-3202 and sodium acetate in the modified Sturm test

The relative biodegradation values calculated from the measurements performed during the test period revealed 50% and 46% biodegradation of MLA-3202 (based on ThCO₂), for the duplicate bottles tested. Thus, the criterion for ready biodegradability (at least 60% biodegradation within a 10-day window) was not met.

In the toxicity control, more than 25% biodegradation occurred within 14 days (50%, based on ThCO₂). Therefore, the test item was assumed not to inhibit microbial activity.

Functioning of the test system was checked by testing the reference item sodium acetate, which showed a normal biodegradation curve (see also paragraph [5.7.2](#)).

7.3. Monitoring of temperature and pH

The temperature recorded in a vessel with water in the same room varied between 21.4 and 22.9 °C.

The pH values of the different test media are presented in [Table 2](#).

Table 2
pH values of different test media.

Test medium:	At the start of the test:	On day 28:
Blank control (A)	7.5	7.6
Blank control (B)	7.5	7.6
Positive control	7.5	8.0
MLA-3202 (A)	7.7 → 7.6 ¹	7.4
MLA-3202 (B)	7.6	7.6
Toxicity control	7.6	7.9

¹: Adjusted using 1 M HCl (Merck, Darmstadt, Germany)

8. CONCLUSION

MLA-3202 was biodegraded significantly (50% and 46%) during the test period. However, since at least 60% biodegradation was not reached within 10 days immediately following the attainment of 10% biodegradation (10-day window), the criterion for ready biodegradability was not met. Thus, under the conditions of this test MLA-3202 was not readily biodegradable.

However, it is expected that MLA-3202 will be inherently biodegradable, as an inherent biodegradability test has more optimal conditions than a ready test.

APPENDIX 1
TABLES

Notes: Except for the percentages biodegradation, all calculations are performed without rounding off.

Produced CO₂: negative values are expressed as 0.00 mL HCl.

Table 3
HCl titrated in duplicate blank bottles

Day	HCl (0.05 M) titrated (mL)		
	Blank A	Blank B	Mean Value
2	47.40	45.97	46.69
5	45.70	45.43	45.57
7	45.28	44.30	44.79
9	45.84	45.50	45.67
14	44.41	44.20	44.31
19	43.57	43.66	43.62
23	42.70	42.46	42.58
27	43.60	43.31	43.46
29	43.16	42.41	42.79
29	46.45	45.82	46.14
29	48.38	48.21	48.30

Table 4
HCl titrated in Ba(OH)₂ solution (background bottles)

Day	HCl (0.05 M) titrated (mL)		
	Bottle A	Bottle B	Mean value
2	49.48	49.54	49.51
5	49.97	49.32	49.65
7	48.92	48.55	48.74
9	48.83	48.71	48.77
14	49.21	48.99	49.10
19	49.24	49.33	49.29
23	49.01	49.51	49.26
27	50.00	50.00	50.00
29	49.54	48.89	49.22
29	49.43	49.80	49.62
29	49.75	49.46	49.61

APPENDIX 1
TABLES– CONTINUED

Table 5
CO₂ production in the blank.

Day	HCl (0.05 M) titrated (mL)		Produced CO ₂ (mL HCl)	Produced CO ₂ (mg)	Cumulative CO ₂ (mg)
	Ba(OH) ₂ ¹⁾	Blank (mean)			
2	49.51	46.69	2.83	3.1	3.1
5	49.65	45.57	4.08	4.5	7.6
7	48.74	44.79	3.95	4.3	11.9
9	48.77	45.67	3.10	3.4	15.3
14	49.10	44.31	4.80	5.3	20.6
19	49.29	43.62	5.67	6.2	26.9
23	49.26	42.58	6.68	7.3	34.2
27	50.00	43.46	6.55	7.2	41.4
29	49.22	42.79	6.43	7.1	48.5
29	49.62	46.14	3.48	3.8	52.3
29	49.61	48.30	1.31	1.4	53.7

¹⁾: "Strength" of untreated 0.0125 M Ba(OH)₂ solution

Table 6
CO₂ production and percentage biodegradation of the positive control item.

Day	HCl (0.05 M) titrated (mL)		Produced CO ₂ (mL HCl)	Produced CO ₂ (mg)	Cumulative CO ₂ (mg)	Biodegradation ¹⁾ (%)
	Blank (mean)	Positive control				
2	46.69	45.56	1.13	1.2	1.2	1
5	45.57	23.80	21.77	23.9	25.2	29
7	44.79	30.22	14.57	16.0	41.2	48
9	45.67	36.61	9.06	10.0	51.2	60
14	44.31	33.63	10.68	11.7	62.9	73

¹⁾: Calculated as the ratio between CO₂ produced (cumulative) and the ThCO₂ of sodium acetate: 85.9 mg CO₂/2L

Table 7
CO₂ production and percentage biodegradation of the test item (bottle A).

Day	HCl (0.05 M) titrated (mL)		Produced CO ₂ (mL HCl)	Produced CO ₂ (mg)	Cumulative CO ₂ (mg)	Biodegradation ¹⁾ (%)
	Blank (mean)	Bottle A				
2	46.69	45.66	1.03	1.1	1.1	1
5	45.57	40.43	5.14	5.6	6.8	8
7	44.79	36.62	8.17	9.0	15.8	18
9	45.67	39.97	5.70	6.3	22.0	25
14	44.31	37.71	6.60	7.3	29.3	33
19	43.62	40.17	3.44	3.8	33.1	38
23	42.58	37.21	5.37	5.9	39.0	44
27	43.46	40.30	3.16	3.5	42.5	48
29	42.79	41.44	1.35	1.5	43.9	50
29	46.14	46.00	0.14	0.1	44.1	50
29	48.30	48.35	0.00	0.0	44.1	50

¹⁾: Calculated as the ratio between CO₂ produced (cumulative) and the ThCO₂ of the test item: 87.7 mg CO₂/2L

APPENDIX 1
TABLES– CONTINUED

Table 8**CO₂ production and percentage biodegradation of the test item (bottle B).**

Day	HCl (0.05 M) titrated (mL)		Produced CO ₂ (mL HCl)	Produced CO ₂ (mg)	Cumulative CO ₂ (mg)	Biodegradation ¹⁾ (%)
	Blank (mean)	Bottle B				
2	46.69	45.34	1.35	1.5	1.5	2
5	45.57	37.84	7.72	8.5	10.0	11
7	44.79	39.38	5.41	6.0	15.9	18
9	45.67	40.70	4.97	5.5	21.4	24
14	44.31	34.11	10.20	11.2	32.6	37
19	43.62	39.98	3.64	4.0	36.6	42
23	42.58	40.89	1.69	1.9	38.5	44
27	43.46	41.85	1.61	1.8	40.2	46
29	42.79	43.20	0.00	0.0	40.2	46
29	46.14	46.90	0.00	0.0	40.2	46
29	48.30	48.56	0.00	0.0	40.2	46

¹⁾: Calculated as the ratio between CO₂ produced (cumulative) and the ThCO₂ of the test item: 87.7 mg CO₂/2L**Table 9****CO₂ production and percentage biodegradation of the toxicity control.**

Day	HCl (0.05 M) titrated (mL)		Produced CO ₂ (mL HCl)	Produced CO ₂ (mg)	Cumulative CO ₂ (mg)	Biodegradation ¹⁾ (%)
	Blank (mean)	Toxicity control				
2	46.69	39.94	6.75	7.4	7.4	4
5	45.57	20.65	24.92	27.4	34.8	20
7	44.79	30.71	14.08	15.5	50.3	29
9	45.67	34.16	11.51	12.7	63.0	36
14	44.31	22.98	21.33	23.5	86.4	50

¹⁾: Calculated as the ratio between CO₂ produced (cumulative) and the sum of the ThCO₂ of the test item and positive control: 174.2 mg CO₂/2L (ThCO₂ test item: 88.2 mg CO₂/2L + ThCO₂ sodium acetate: 85.9 mg CO₂/2L)**Table 10****Comparison of biodegradation of the test item in bottles A and B.**

Day	Biodegradation (%)			
	Bottle A	Bottle B	Mean A and B	Δ A-B ¹⁾
2	1	2	2	1
5	8	11	10	3
7	18	18	18	0
9	25	24	25	1
14	33	37	35	4
19	38	42	40	4
23	44	44	44	0
27	48	46	47	2
29	50	46	48	4
29	50	46	48	4
29	50	46	48	4

¹⁾: Absolute difference in biodegradation between bottles A and B

APPENDIX 2
CERTIFICATE OF ANALYSIS



Chempura Corporation
 12 Spencer St
 Naugatuck, CT 06770

Analytical Services
www.chemtura.com

Certificate of Purity

Customer: Support for Toxicology Studies

Test Substance Name: MLA3202; Amides, tallow, N,N-bis(2-hydroxypropyl)

Physical Appearance: Liquid

CAS No.: 1454803-04-3

Ref. or Lot Number: RC-1045

Date of Analysis: revised March 18, 2016 (original issue March 7, 2016)

Percent Composition	Monoisotopic Mass (daltons)	Formula	Structure/ Identity
33.1	397.4	C ₂₄ H ₄₇ NO ₃	C18:1 (oleic) tallow amides, N,N-bis(2-hydroxypropyl)
22.9	371.3	C ₂₂ H ₄₅ NO ₃	C16:0 (palmitic) tallow amides, N,N-bis(2-hydroxypropyl)
13.6	395.4	C ₂₄ H ₄₅ NO ₃	C18:2 (linoleic) tallow amides, N,N-bis(2-hydroxypropyl)
11.0	399.4	C ₂₄ H ₄₉ NO ₃	C18:0 (stearic) tallow amides, N,N-bis(2-hydroxypropyl)
6.0	369.3	C ₂₂ H ₄₃ NO ₃	C16:1 (palmitoleic) tallow amides, N,N-bis(2-hydroxypropyl)
3.2	419.3	C ₂₆ H ₄₅ NO ₃	C20:4 (eicosatetraenoic) tallow amides, N,N-bis (2-hydroxypropyl)
2.0	393.3	C ₂₄ H ₄₃ NO ₃	C18:3 (linolenic) tallow amides, N,N-bis(2-hydroxypropyl)
1.5	282.3	C ₁₈ H ₃₄ O ₂	C18:1 (oleic) acid
1.1	421.4	C ₂₆ H ₄₇ NO ₃	C20:3 (eicosatrienoic) tallow amides, N,N-bis (2-hydroxypropyl)
5.6			Sum of residual components (< 1% each)
100.0			Total

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